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Identification of *Lactobacillus* Isolates from the Gastrointestinal Tract, Silage, and Yoghurt by 16S-23S rRNA Gene Intergenic Spacer Region Sequence Comparisons

G. W. TANNOCK,[1]* A. TILSALA-TIMISJARVI,[2] S. RODTONG,[3] J. NG,[1] K. MUNRO,[1] AND T. ALATOSSAVA[2,4]

Department of Microbiology, University of Otago, Dunedin, New Zealand[1]; Department of Biology, University of Oulu, Oulu,[2] and Biotechnology Laboratory, REDEC of Kajaani, University of Oulu, Sotkamo,[4] Finland; and School of Microbiology, Suranaree University of Technology, Nakhon Ratchasima, Thailand[3]

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***Lactobacillus* isolates were identified by PCR amplification and sequencing of the region between the 16S and 23S rRNA genes (spacer region). The sequences obtained from the isolates were compared to those of reference strains held in GenBank. A similarity of 97.5% or greater was considered to provide identification. To check the reliability of the method, the V2-V3 region of the 16S rRNA gene was amplified and sequenced in the case of isolates whose spacer region sequences were less than 99% similar to that of a reference strain. Confirmation of identity was obtained in all instances. Spacer region sequencing provided rapid and accurate identification of *Lactobacillus* isolates obtained from gastrointestinal, yoghurt, and silage samples. It had an advantage over 16S V2-V3 sequence comparisons because it distinguished between isolates of *Lactobacillus casei* and *Lactobacillus rhamnosus*.**

The members of the genus *Lactobacillus* are gram-positive organisms that belong to the general category of lactic acid bacteria. They inhabit a wide variety of habitats, including the gastrointestinal tracts of animals and vegetation, and are used in the manufacture of fermented foods (8). Interest in the lactobacilli has been stimulated in recent years by the use of these bacteria in products that are claimed to confer health benefits on the consumer (probiotics) (4).

The identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) (8). In general, about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level (6). The derivation of simple yet rapid identification methods is therefore required in order to deal with the large numbers of *Lactobacillus* isolates obtained during microbial ecological studies of ecosystems such as the intestinal tract, silage, and food products.

Nucleotide base sequences of *Lactobacillus* 16S ribosomal DNA (rDNA) provide an accurate basis for phylogenetic analysis and identification (2, 5, 17). The sequence obtained from an isolate can be compared to those of *Lactobacillus* species held in data banks. Although the species-specific sequences are contained in the first half of the 16S rRNA gene (V1-V3 region), identification is more accurate if the whole gene is sequenced (13). This means that about 1.5 kb of DNA would have to be sequenced.

Studies by Tilsala-Timisjarvi and Alatossava (15), Berthier and Ehrlich (3), Nour (11), and Nakagawa and colleagues (10) have demonstrated that the DNA sequence between the 16S and 23S genes of lactobacilli is hypervariable. This intergenic spacer region is about 200 bases in length if tRNA genes are absent (small spacer sequence) (3). The 16S-23S spacer sequences of lactobacilli are sufficiently species specific for the derivation of PCR primers that can be used to identify *Lactobacillus* species (3, 10, 15).

Because a relatively large number of different species (at least 18 from monogastric animals) have been described as intestinal inhabitants (9), identification of lactobacilli by PCR using sets of specific primers is daunting logistically. We have therefore sequenced the 16S-23S small spacer regions of *Lactobacillus* isolates and compared them to the sequences of type cultures and other valid strains recorded in GenBank (National Center for Biotechnology Information, Bethesda, Md.). Our results show that this is a relatively simple and rapid method by which lactobacilli can be identified without resorting to the use of species-specific PCR primers.

Twenty-eight intestinal isolates (from human feces, rodent gastrointestinal samples, and porcine gastrointestinal contents), 10 isolates from probiotic yoghurts retailed in Dunedin supermarkets, and 2 silage isolates from Thailand were used in the study (Table 1). They were determined to belong to the genus *Lactobacillus* by culture on Rogosa SL agar (Difco Laboratories, Detroit, Mich.), Gram stain appearance, catalase test, and determination of fermentation products by gas-liquid chromatography (7). The 16S-23S intergenic spacer region from each isolate was amplified by using primers (15) that annealed to conserved regions of the 16S and 23S genes (primer 16-1A, 5′-GAATCGCTAGTAATCG-3′, corresponding to nucleotides 1361 to 1380 of the 16S rRNA gene according to *Lactobacillus casei* numbering [18]; primer 23-1B, 5′-GGGTT CCCCCATTCGGA-3′, corresponding to nucleotides 123 to 113 of the 23S rRNA of *L. casei* [10]). PCR mixtures contained 5 μl of 10× polymerase buffer (Boehringer Mannheim GmbH, Mannheim, Germany), 200 μM each deoxynucleoside triphosphate, 80 pM each primer, 2 μl of *Lactobacillus* cell suspension (a few colonies emulsified in sterile MilliQ [Millipore Corp.] water), and 2.6 U of Expand High Fidelity PCR System (Boehringer Mannheim) DNA polymerase in a total volume of

* Corresponding author. Mailing address: Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand. Phone: 64-3-479-7713. Fax: 64-3-479-8540. E-mail: gerald.tannock@stonebow.otago.ac.nz.

TABLE 1. *Lactobacillus* isolates identified on the basis of percent similarity to 16S-23S small spacer region sequences in GenBank

Isolate	Source	GenBank accession no. of 16S-23S sequence	Species identification based on 16S-23S sequence	% Similarity of 16S-23S sequence to that of reference strains in GenBank	GenBank accession no. of V2-V3 sequence	Species identification based on sequence of 16S V2-V3 region
GTH1	Human	AF158557	*L. salivarius* subsp. *salivarius*	100.0	NA[a]	ND[b]
GTH2	Human	AF158558	Unidentified	NA	AF157030	*W. confusa*
GTH5	Human	AF158559	*L. gasseri*	98.6	AF157044	*L. gasseri*
GTH6	Human	AF158560	*L. gasseri*	98.2	AF157033	*L. gasseri*
GTH7	Human	AF158561	*L. ruminis*	99.5	NA	ND
GTH8	Human	AF158562	Unidentified	NA	AF157045	*W. confusa*
GTH10	Human	AF158563	*L. fermentum*	99.0	NA	ND
GTH13	Human	AF158564	*L. rhamnosus*	99.5	NA	ND
GTH15	Human	AF158565	*L. casei*	99.5	AF157043	*L. casei* group
GTH17	Human	AF158566	*L. rhamnosus*	99.5	NA	ND
GTH18	Human	AF158567	*L. brevis*	98.2	AF157038	*L. brevis*
GTH20	Human	AF158568	*L. rhamnosus*	99.5	NA	ND
GTH22	Human	AF158569	Unidentified	NA	AF157037	*W. confusa*
GTH24	Human	AF158570	Unidentified	NA	AF157031	*W. confusa*
GTH26	Human	AF158571	Unidentified	NA	AF157034	*W. confusa*
GTH28	Human	AF158572	*L. ruminis*	98.6	AF157032	*L. ruminis*
GTH29	Human	AF158573	*L. gasseri*	98.6	AF157039	*L. gasseri*
GTH30	Human	AF158574	*L. casei*	99.1	AF157042	*L. casei* group
GTH32	Human	AF158575	*L. rhamnosus*	100.0	NA	ND
GTH33	Human	AF158576	*L. fermentum*	99.5	NA	ND
JNI	Yoghurt	AF158577	*L. delbrueckii* subsp. *bulgaricus*	98.5	AF157040	*L. delbrueckii*
JN2	Yoghurt	AF158578	*L. delbrueckii* subsp. *bulgaricus*	99.5	NA	ND
JN3	Yoghurt	AF158579	*L. helveticus*	99.5	NA	ND
JN4	Yoghurt	AF158580	*L. delbrueckii* subsp. *bulgaricus*	98.6	AF157041	*L. delbrueckii*
JN5	Yoghurt	AF158581	*L. acidophilus*	100.0	NA	ND
JN6	Yoghurt	AF158582	*L. acidophilus*	100.0	NA	ND
JN7	Yoghurt	AF158583	*L. acidophilus*	100.0	NA	ND
JN8	Yoghurt	AF158584	*L. helveticus*	99.5	NA	ND
JN9	Yoghurt	AF158585	*L. acidophilus*	100.0	NA	ND
JN10	Yoghurt	AF158586	*L. rhamnosus*	100.0	NA	ND
GTP1	Pig	AF158587	*L. crispatus*	100.0	NA	ND
GTP2	Pig	AF158588	*L. crispatus*	100.0	NA	ND
GTP3	Pig	AF158589	*L. crispatus*	100.0	NA	ND
GTP4	Pig	AF158590	*L. reuteri*	100.0	NA	ND
GTP5	Pig	AF158591	*L. crispatus*	98.5	AF157035	*L. crispatus*/*L. gallinarum*
GTP6	Pig	AF158592	*L. crispatus*	100.0	NA	ND
SR1	Silage	AF158593	*L. pentosus*/*L. plantarum*	99.5	NA	ND
SR2	Silage	AF158594	*L. pentosus*/*L. plantarum*	97.5	AF157036	*L. pentosus*/*L. plantarum*
GTR1	Mouse	AF158595	*L. reuteri*	100.0	NA	ND
GTR3	Rat	AF158596	*L. reuteri*	100.0	NA	ND

[a] NA, not applicable.
[b] ND, not done.

50 μl. The PCR program began with a preincubation at 94°C for 2 min. The amplification profile was 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. This was repeated for 30 cycles. The program finished with a 5-min incubation at 72°C. PCR products were electrophoresed in a 1% agarose gel and visualized by UV transillumination after being stained in ethidium bromide solution (5 μg/ml). *Lactobacillus* species frequently contain both small and large spacer regions, in which case the smallest product (about 500 to 600 bp) was excised from the gel and extracted by using a QIAEX kit (Qiagen, Hilden, Germany). The extract was used in a repeat PCR amplification, and the resulting DNA was purified from primers and unincorporated nucleotides by using a QIAquick kit (Qiagen). Both polynucleotide strands of the purified DNA were sequenced, using 16-1A and 23-1B as forward and reverse primers, respectively. Sequencing was carried out either at the Centre for Gene Research, University of Otago, by the dideoxy method of Sanger et al. (12), using a PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., Foster City, Calif.) in combination with an Applied Biosystems model 377A automated sequencing system, or manually by using a Circum Vent Thermal Cycle Dideoxy kit (New England Biolabs, Beverly, Mass.). Analysis of nucleotide sequence data was carried out by using the SeqEd program, version 1.0.3 (Applied Biosystems Inc.). Further sequence editing and analysis were carried out with either EditSeq version 3.98 (DNA Star Inc., Madison, Wis.) and Megalign version 3.1.7 (DNA Star Inc.) or LKB DNASIS (version 7.0). The small intergenic spacer region sequences obtained by these methods were compared to sequences from type culture and other *Lactobacillus* strains held in GenBank (Table 2).

To test the reliability of the method, we extracted DNA from *L. acidophilus* ATCC 4356^T on five separate occasions and sequenced the amplified 16S-23S spacer region. The five sequences were 100.0% similar to each other, as well as to the sequence held in GenBank.

Comparisons of 16S-23S small spacer region sequences held in GenBank showed that all were less than 97.5% similar except for *L. salivarius* subsp. *salivarius* and *L. salivarius* subsp. *salicinius* (98.6% similar), *L. plantarum* and *L. pentosus* (99.0% similar), and *L. curvatus* and *L. graminis* (98.6% similar). With these exceptions, in which small spacer region sequences can only aid in grouping isolates, we arbitrarily chose a value for similarity between sequences of 97.5% or greater to indicate

TABLE 2. GenBank *Lactobacillus* 16S-23S intergenic spacer region sequences used to identify isolates

Species	Strain[a]	GenBank accession no.
L. acidophilus	ATCC 4356^T	U32971
L. brevis	IFO 13110	X74221
L. casei	ATCC 334^T	AF121200
L. crispatus	ATCC 33820^T	AF074857
L. curvatus	DSM 20019^T	U97129
L. delbrueckii subsp. *bulgaricus*	ATCC 11842^T	AF113602
L. delbrueckii subsp. *lactis*	ATCC 15808	U32968
L. fermentum	ATCC 14931^T	AF080099
L. gasseri	ATCC 33323^T	AF074859
L. graminis	DSM 20719^T	U97130
L. hamsteri	ATCC 43851^T	AF113601
L. helveticus	ATCC 15009^T	U32970
L. johnsonii	ATCC 33200^T	AF074860
L. pentosus	ATCC 8041^T	U97134
L. plantarum	ATCC 14917^T	AF080101
L. reuteri	DSM 20016^T	AF080100
L. rhamnosus	ATCC 7469^T	AF121201
L. ruminis	ATCC 277780^T	AF080103
L. sake	ATCC 15521^T	U97131
L. salivarius subsp. *salicinius*	ATCC 11742^T	AF080102
L. salivarius subsp. *salivarius*	ATCC 11741^T	AF113600
L. sharpeae	DSM 20505^T	AF074861
L. zeae	ATCC 393^T	AF074862

[a] ATCC, American Type Culture Collection, Manassas, Va.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; superscripted T, type culture.

species identity. This cutoff value is supported by the observation that in cases where 16S rDNA sequence (whole gene) homologies are below about 97%, it is unlikely that two organisms have more than 60 to 70% DNA similarity and, hence, that they are related at the species level (13).

Comparisons of 16S-23S small spacer region sequences obtained from *Lactobacillus* isolates with those held in GenBank enabled the identification of 35 of 40 isolates (Table 1). Intergenic spacer region sequences from isolates GTH2, GTH8, GTH22, GTH24, and GTH26 did not correspond to sequences in the data bank. Nine isolates (GTH5, GTH6, GTH18, GTH28, GTH29, JN1, JN4, GTP5, and SR2) had spacer sequences that were between 97.5 and 99.0% similar to sequences in the database. To confirm that the cutoff similarity value used (97.5%) was valid, we amplified and sequenced (one polynucleotide strand only) the V2-V3 regions of the 16S rRNA genes of these isolates and conducted a search of sequences deposited in the GenBank DNA database by using the BLAST algorithm (1). Amplification of the V2-V3 region was accomplished by using primers HDA1 (5′-ACTCCTACGGG AGGCAGCAGT-3′) and HDA2 (5′-GTATTACCGCGGCT GCTGGCAC-3′), described by Turner et al. (16), and the thermal cycler program described above. The PCR product corresponded to positions 339 to 539 in the *Escherichia coli* gene. BLAST searches confirmed the identities (on the basis of highest score) obtained by spacer sequence analysis of all nine isolates and identified isolates GTH8, GTH26, GTH2, GTH22, and GTH24 as *Weissella confusa* (previously known as *Lactobacillus confusus*), for which the 16S-23S spacer region of the type culture is not yet available (Table 1). Additionally, we sequenced the V2-V3 regions of GTH15 and GTH30, which had been identified as *L. casei* on the basis of spacer region sequence. The highest scores for the V2-V3 sequence, for both isolates, were obtained for members of the *L. casei* group (*L. casei*, *L. paracasei*, and *L. rhamnosus*).

Our study shows that comparison of the percentages of similarity of 16S-23S spacer region sequences of lactobacilli provides a practical method of strain identification. The small 16S-23S spacer sequences of lactobacilli are about 200 bp in length. These relatively short sequences can be easily sequenced on both polynucleotide strands and provide reliable information for comparative work. The spacer sequence identifications that showed less than 99.0% similarity to those of reference strains were confirmed in all cases by sequencing the V2-V3 regions of their 16S rDNAs. Moreover, the spacer region method had the advantage of distinguishing between *L. rhamnosus* and *L. casei* strains, which cannot be accomplished by comparison of 16S V2-V3 region sequences. These species are commonly used in the production of probiotic products (14). As we have demonstrated here, amplification of the spacer regions by PCR can be carried out with suspensions of whole *Lactobacillus* cells, so colonies picked from agar plates can be used directly in identification of an isolate. DNA of a quality suitable for sequencing can be obtained within 48 h of culture of lactobacilli. For the majority of our *Lactobacillus* isolates, a clear species identification could be made on the basis of percent similarity to GenBank sequences (97.5 to 100.0% similarity). Even when 16S-23S sequences do not differ greatly between species (e.g., the *L. pentosus*/*L. plantarum* group), identification is at least aided by grouping of the isolate, as was the case with the silage strains. The use of 16S-23S spacer sequences in the identification of lactobacilli promises to be a valuable aid in advancing our knowledge of the species composition of *Lactobacillus* populations.

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